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REVIEW

# The Pathway of Sperm-Egg Interaction in Ascidians: Biology and Chemistry

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## INTRODUCTION

Marine organisms have been the elective material for the understanding of many challenging problems in biology. This is particularly the case of the sea urchin, which since the second half of 19th century, has played a key role in the field of developmental biology, equaled only by amphibians.

At the beginning of the century another model was proposed by E. D. Conklin. He described in detail the organization and cell-lineage of the ascidian egg, indicating that ascidians provide one of the best examples for the study of cell line segregation [1]. Conklin's comprehensive work raised a series of questions on different and also temporally distant events, paving the way for the studies that were to follow: along this line, T. H. Morgan resumed studies of the early stages of sperm-egg interaction and, in particular, that of the genetic regulation of self-sterility in *Ciona intestinalis* [2]. In fact ascidians are hermaphroditic and, to some extent, self-sterile animals, hence they offer a unique opportunity to study the problem of self-nonself recognition. Morgan suggested that in *Ciona intestinalis* the self-sterility barrier is under the control of more than one allele and that it resides in the egg investments [2]. These observations have been confirmed and extended: in fact it has been found that this barrier resides in the vitelline coat (VC) which modulates the self-sterility [3–5]. Since Morgan's observations, a considerable amount of data from several laboratories has shown that in ascidians, species specific binding of spermatozoa occurs on the egg extracellular coat, the VC, and that binding is a key step in fertilization, preliminary to all the events leading to gamete fusion [5–8]. Recently efforts have been made to isolate the molecules of the vitelline coat responsible for the sperm binding: these molecules have been also characterized both biochemically and functionally [9–11].

Few studies have been conducted on the counterpart of the VC on the spermatozoon. Most of the data available deal with the structure and the physiology of this cell in relation to the binding process [12–19]. Recently studies have been undertaken aimed at identifying the molecules involved in the interaction and at clarifying the mechanism underlying these events [7, 9, 10, 12, 20–23].

In this paper we review studies on the early stages of sperm-egg interaction in the ascidians with special attention to the biology and chemistry of the binding. Similarities with sea urchin and mammal fertilization are also touched upon.

## THE MAIN EVENTS IN SPERM-EGG INTERACTION

A general and simplified scheme of the events leading to the penetration of spermatozoa into the

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perivitelline space can be drawn from observations carried out in *Ciona intestinalis*: the spermatozoon passes between the follicle cells and binds firmly to the VC. It is activated, it undergoes the acrosome reaction and then penetrates through the VC.

Even though follicle cells are the first barrier the spermatozoon encounters in approaching the egg, they do not seem to be necessary for a successful interaction [6]. A chemotactic role for the follicle cells has been proposed by Miller, who examined several ascidian species [23, 24]. This observation has been confirmed in *Phallusia* and in *Asidia nigra*, in which removal of the follicle cells results in a slight reduction of the fertilization rate [7, 25]. So far, only in the Japanese ascidian *Halocynthia roretzi* the follicle cells seem to be strictly necessary for fertilization [3].

The spermatozoon reaches the VC and within a few minutes binds firmly and species-specifically to the outer surface of the coat by the plasma membrane of its tip. Binding occurs at the whole surface of the VC not covered by the follicle cells, apparently without any polarity [5–8, 18]. In ascidians binding is neither preceded by a loose and nonspecific "attachment", as described in the mouse, nor followed by a cortical reaction, as in mammals and echinoderms.

Upon binding, the spermatozoon undergoes a series of changes, that can be referred to as sperm "reaction" or "activation" [16, 26]: these consist of the swelling and translocation of the cylindrical mitochondrion towards the tail and of concomitant physiological changes. The reaction culminates in the exocytosis of the vesicle/s present at the sperm tip, which is interpreted as an acrosome reaction [6, 7, 19]. The acrosome reaction leads to the penetration of the spermatozoon through the VC. Trypsin-like and chymotrypsin-like proteases contained in the vesicles seem to be the lytic agents that allow the spermatozoon to penetrate through the VC [7, 27–30].

The spermatozoon passes between the test cells apparently unimpeded by these cells. Since no tool has been found whereby the activity of these cells can be discriminated from that of the VC, only indirect experimental evidence is available. Hence, even though test cells seem not to have a primary role, their participation in the interaction cannot be ruled out [3].

Once the spermatozoon has crossed this last barrier, it makes contact with the egg plasma membrane, on which fusion occurs at the vegetal pole [4, 31].

## SPERM-EGG BINDING

Most of the data about sperm-egg interaction in ascidians concern the binding of the spermatozoa to the VC, a preliminary and essential step for species-specific recognition between the gametes.

Binding is established between the tip of sperm head and fibrils emerging from the outer surface of the VC [6, 7]. Screening of the surface of the VC with lectins and the effect of correspondent competitive sugars on binding and fertilization revealed that in *Ciona intestinalis*, egg fucosyl sites present on the fibrils have a key role in sperm binding [32, 33]. In *Phallusia mammillata* and in *Ascidia nigra*, N-acetylglucosamine residues present on the VC have the same function in sperm binding [7, 8, 34]. The biochemical and functional characterizations of the molecules involved in this interaction have been conducted mainly on *Ciona intestinalis*. A glycoprotein complex with sperm receptor activity has been isolated from the VC of the mature egg. SDS-PAGE analysis of this complex followed by incubation of the gel with $^3$H-*Lotus tetragonolobus* agglutinin (LTA) revealed the presence of five fucosyl-containing glycoproteins (FP). FP exhibited the properties expected for the receptor: i.e. they inhibited binding of the spermatozoa to the VC and fertilization. Furthermore, spermatozoa incubated with FP were activated and triggered into the acrosome reaction [9]. FP are synthesized by the oocyte during the vitellogenic stages of oogenesis [35]. This demonstrates that the glycoprotein complex with sperm receptor activity, on which the fine tuning and in fact the success of fertilization depend, is synthesized under the control of the oocyte genome.

These findings led to a further biochemical and functional analysis of FP purified by affinity chromatography from the ovary [10]. The separation by gel filtration of the fucosyl glycoproteins into the units identified by SDS-PAGE, always resulted in the elution of FP with the void volume, thus indicating that the sperm receptor on the VC is a highly complex glycoconjugate with an extraordinarily high apparent molecular weight ($>10^7$ D) and a protein-carbohydrate ratio of 2: 1. FP inhibit the binding of the spermatozoa to the VC and induce sperm activation and the acrosome reaction [10]. Exhaustive proteolytic digestion of FP yields high molecular weight glycopeptides ($>4\times10^5$), which contain N-acetylgalactosamine, fucose, galactose and rhamnose. These glycopeptides, when compared with the intact FP, have much lower capacity to inhibit sperm binding, but they do not induce sperm activation and, in consequence, the acrosome reaction [10]. These findings raise the question of the involvement of the polypeptide component in the sperm-egg binding process, either directly or as a carrier of the carbohydrate chains. In the latter case, the polypeptide chain could provide a backbone for the specific assembly or conformation of the carbohydrate component, thus allowing the most effective molecular match with the counterpart on the spermatozoon [10].

Hence, FP share many of the characteristics of both the sperm receptors of the egg of *Strongylocentrotus purpuratus* [36] and of the zona pellucida of the mouse [37]. In the first case, in fact, the receptor for the spermatozoa is a high molecular weight proteoglycan-like molecule (greater than $10^7$) that is responsible for the species specific binding of the spermatozoa to the egg. As to the biological activity, FP are more similar to ZP3, the zona pellucida glycoprotein of the mouse egg, which serves as both receptor for spermatozoa and inducer of the sperm acrosome reaction. Also in this case the sperm receptor activity is dependent only on its carbohydrate component, whereas the whole complex is responsible for the acrosome reaction-inducing activity [37].

While the sperm receptors on the egg have been isolated and extensively characterized in several systems, their counterparts on the spermatozoon and the mechanisms underlying the sperm-egg interaction have yet to be elucidated. In the sea urchin, upon contact with the egg jelly through a sperm plasma membrane glycoprotein [38], the spermatozoon undergoes the acrosome reaction, thus exposing “bindin”, a protein associated with the inner acrosomal membrane [39]. Bindin interacts species-specifically with the glycoprotein sperm receptor on the VC by a lectin-polysaccharide type of interaction [40]. In mouse, it has been suggested that different kinds of proteins associated with the sperm plasma membrane participate in the sperm-zona interaction [37]. A galactosyltransferase localized at the sperm surface is at least one of the components involved in the sperm binding to the egg zona pellucida through an enzyme-substrate mechanism [41, 42].

Also in *Ciona intestinalis* there is evidence that different proteins present on the surface of the spermatozoa are involved in the sperm-egg interaction. Concanavalin A-binding sites are present at the tip of the sperm head: these glycoproteins have been isolated by affinity chromatography from the tip of the sperm head and they proved to be active in the inhibition of binding [20].

Furthermore, it has been suggested that an $\alpha$-L-fucosidase, isolated from *Ciona* spermatozoa is involved in the very early stages of binding [21, 43]. This enzyme would interact with the fucosylglycoproteins of the VC by forming an enzyme-substrate complex. Whether this interaction proceeds with the hydrolysis of fucose, has yet to be elucidated. In *Phallusia mammillata* where terminal N-acetylglucosamine is functional in sperm binding [7, 34], it has been found that a $\beta$-D-N-acetylglucosaminidase retains the highest activity among sperm glycosidases [21, 43]. These findings support the hypothesis that in different systems glycoproteins of the egg envelope and carbohydrate-binding proteins of the spermatozoon mediate the sperm-egg recognition and binding. These proteins can be roughly classified into two groups: enzymes, such as the galactosyltransferase of mouse and the $\alpha$-L-fucosidase of ascidians, and lectin-like proteins, such as the “bindin” of the sea urchin spermatozoon.

Indeed, the sperm-egg interaction proceeds through a far more complicated series of connected and ordered reactions, that involve different molecules and/or different functional sites on the same molecule [37, 44, 45]. In fact, in mouse there is evidence that, besides galactosyltransferase, also a trypsin-like proteinase may be involved in binding [44, 46]. Furthermore, a fucose-binding protein isolated from boar spermatozoa has been demonstrated to be identical with the sperm proteinase acrosin. This molecule combines specific proteolytic activity with zona- and carbohydrate-affinity properties, thus suggesting that the lectin-like activity of the acrosin directs the proteolytic activity to its structural target [45].

## SPERM ACTIVATION, ACROSOME REACTION AND PENETRATION

Upon binding to the VC ascidian spermatozoon undergoes a number of morphological and physiological changes called "sperm reaction" [16, 26]. Spermatozoa lack a midpiece and they have a single cylindrical mitochondrion alongside the nucleus in the head. When the spermatozoon binds to the VC by the tip of the head, the mitochondrion swells, becomes spherical and starts sliding towards the tail. Occasionally, the mitochondrion may even be cast off. This reaction has been also observed in spermatozoa bound to isolated VC [16] or can be induced by the calcium ionophores [14, 22]. Since the presence of actin and myosin has been revealed by indirect immunofluorescence in the spermatozoa of several species of ascidians, it has been proposed that actin-myosin sliding is responsible for mitochondrion translocation [18]. In the sperm traversing the VC and the perivitelline space, the membrane of the spermatozoon is anchored to the VC by the outer surface overlying the mitochondrion and to the mitochondrion by its inner surface. As the mitochondrion moves along the tail, the sperm is driven across the perivitelline space. When the mitochondrion reaches the tip of the tail, it is released.

Sperm reaction is accompanied by a release of $H^+$; this causes intracellular alkalinization, which in turn increases permeability to calcium [22, 47]. Hence, the onset of binding might trigger the activation of calcium channels that promotes the uptake of $Ca^{2+}$ into spermatozoa and the acrosome reaction.

Changes at the tip of the spermatozoa referred to as an acrosome reaction have been described only in *Ciona intestinalis* and in *Phallusia mammillata* [6, 7, 19]. The reaction proceeds through the exocytosis of one or some acrosomal vesicles of the spermatozoa just about to penetrate the VC. It can be inferred that the acrosomal vesicles contain lysins necessary for the penetration of the spermatozoa through the VC [19]. In fact, a preliminary screening to detect protease activity involved in sperm penetration produced evidence indicating that in Pleurogona both chymotrypsin- and trypsin-like activities are necessary, while in Enterogona only the chymotrypsin-like activity is required for the penetration through the VC [30, 43, 48]. More recently, two types of trypsin-like proteases have been purified from spermatozoa of *Halocynthia roretzi* [28]. Whereas one of the enzymes showed properties closely related to those of mammalian acrosin, the other seemed to be a novel type of sperm acrosin-like enzyme with very strict substrate specificity. These enzymes, named ascidian acrosin and spermosin respectively, exerted lytic activity on the coat only together with chymotrypsin-like enzyme(s). It was suggested that ascidian spermosin, acrosin and chymotrypsin-like enzyme are involved in a "lysin system" [28]: the timing of action of the three sperm proteases was examined by adding specific protease inhibitors at various times after insemination [29]. It seems likely that spermosin and the chymotrypsin-like enzyme function at an early stage of the process of penetration through the egg investment, while acrosin functions at a later stage [29].

Concerning the number of spermatozoa that cross the VC, it is generally agreed that rare acrosome-reacted spermatozoa can be observed on the VC and that "few spermatozoa" can be found within the perivitelline space [4–7]. The suggestion that the VC acts as a barrier for sperm penetration and the apparent discrepancy between the number of spermatozoa bound and those penetrating the VC, indicated that in order for the attachment to be followed by the acrosome reaction there must be a precise "molecular match" between receptors on the egg and the counterpart on the spermatozoon [9].

In ascidians, morphological differences of the VC, as a consequence of the sperm penetration, have never been observed. Only in the Japanese ascidian *Halocynthia roretzi* is there an expansion of the perivitelline space following fertilization, and there is evidence for the participation of a trypsin-like enzyme in this change [49]. By analogy with the role of the trypsin-like enzyme of the sea urchin, this enzyme may function in a limited proteolysis of the VC, which becomes more resistant to proteolysis than the VC of the unfertilized eggs.

A block to polyspermy that requires $Na^+$ has been found in *Ascidia nigra* [8]; this block involves also the release of a protein, either an enzyme or a soluble lectin, that modifies VC N-acetylglucosamine residues required for sperm binding. This in turn causes a rapid decline in the number of sperm bound.

Information about the pathway of the spermatozoon after its passage through the VC is still limited. Most of the data on the steps following penetration concern the site of fusion of the spermatozoon with the egg plasma membrane [4, 31]. Spermatozoa come in contact with the egg plasma membrane and can be found randomly distributed along the animal-vegetal axis of the egg, except at the animal pole [4]. At the time ooplasmic segregation is nearly completed, spermatozoa are localized only at the vegetal pole, where, after fusion, the male pronucleus is located [1, 4]. Sperm-egg fusion occurs between the plasma membrane of the postacrosomal region of the sperm head and the egg plasma membrane [7].

## CONCLUDING REMARKS

The process whereby gametes recognize and adhere to each other is one of the most challenging problems in cell biology. The study of the mechanisms of this interaction showed that the information for establishing species-specific recognition and binding lies in the expression and organization of molecules at the surface of both gametes. In this respect, gamete interaction, although retaining peculiar features, can be regarded as any other cell-cell interaction system [41–52].

In ascidians, as in the vast majority of animals, sperm receptors are glycoproteins assembled in an extracellular envelope, which is a vanguard for the interaction with the spermatozoa. In these organisms the involvement of the carbohydrate and the protein components of the sperm receptors in the binding has to be further elucidated. Concerning the molecular mechanism which regulates binding, a preliminary and essential step of the interaction, there is evidence that glycosidases on the spermatozoa interact with the glycoproteins of the VC by way of an enzyme-substrate mechanism. This mechanism has also been proposed in mouse fertilization and in other cell-cell interaction systems. However, this seems to be an oversimplification of the binding process, in the pathway of which other as yet unidentified molecules may take part. As emerges from this review, knowledge of the complex cascade of events that starts with gamete recognition and leads to the fusion of the pronuclei, has grown in the last decade: however, a fundamental support to the understanding of this process will certainly come from new technologies of molecular biology.

In this context, also one of the most stimulating, and still unsolved, problems in ascidian fertilization, namely the control of self-sterility, will certainly benefit from these technologies. In fact, studies with the colonial ascidian *Botryllus* have suggested that the fine tuning of the gametic self-incompatibility is controlled by genes linked or identical to those that regulate the colony fusibility. It seems likely that gametic and somatic self-recognition genes in protochordates represent ancient functions of primitive major histocompatibility complex genes [53]. A complete understanding of this problem still awaits much more information. What is evident is that in this respect ascidians have proved to be a good example of how the choice of a model can answer more general questions.

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